

# Biotechnological aspects of sulfate reduction with methane as electron donor

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**Abstract** Biological sulfate reduction can be used for the removal and recovery of oxidized sulfur compounds and metals from waste streams. However, the costs of conventional electron donors, like hydrogen and ethanol, limit the application possibilities. Methane from natural gas or biogas would be a more attractive electron donor. Sulfate reduction with methane as electron donor prevails in marine sediments. Recently, several authors succeeded in cultivating the responsible microorganisms *in vitro*. In addition, the process has been studied in bioreactors. These studies have opened up the possibility to use methane as electron donor for sulfate reduction in wastewater and gas treatment. However, the obtained growth rates of the responsible microorganisms are extremely low, which would be a major limitation for applications. Therefore, further research should focus on novel cultivation techniques.

**Keywords** Anaerobic oxidation of methane · Sulfate reduction · Biotechnology · Wastewater treatment

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## 1 Carbon and sulfur cycling in nature

### 1.1 Physical and chemical properties of methane

Methane (CH<sub>4</sub>) is a tetrahedral shaped molecule, and a colorless, nontoxic and odorless gas (above 109°K at 1 atm). CH<sub>4</sub> gas is only flammable when the concentration in the air is between 5 and 15%. It has a relatively low solubility product in water (1.44 mM in distilled water at 20°C and 0.101 MPa CH<sub>4</sub>; Yamamoto et al. 1967). About 2.7 million years ago, CH<sub>4</sub> was a major component in the earth's atmosphere (Chang et al. 1983). Since then, the atmosphere became more oxidized. In 1998, the average atmospheric CH<sub>4</sub> concentration was 1.7 ppm (Houghton et al. 2001). CH<sub>4</sub> is the simplest and most stable hydrocarbon. Compared with other alkanes, CH<sub>4</sub> has a high C–H bond strength, making it chemically rather stable. The dissociation energy of the C–H bond in CH<sub>4</sub> is +439 kJ mol<sup>-1</sup> (Thauer and Shima 2008). CH<sub>4</sub> is the least reactive alkane in reactions involving hydride abstraction by an electrophile, because the C–H bond is not polarized (Crabtree 1995). Therefore, CH<sub>4</sub> is only a good substrate for specialized microorganisms.

CH<sub>4</sub> is the most reduced form of carbon (oxidation state -4), carbon dioxide (CO<sub>2</sub>) being the most oxidized form (oxidation state +4). CH<sub>4</sub> is the main component of natural gas (70–90%) and biogas (50–70%). The energy yield per carbon during oxidation is for CH<sub>4</sub> higher than for other hydrocarbons or coal.

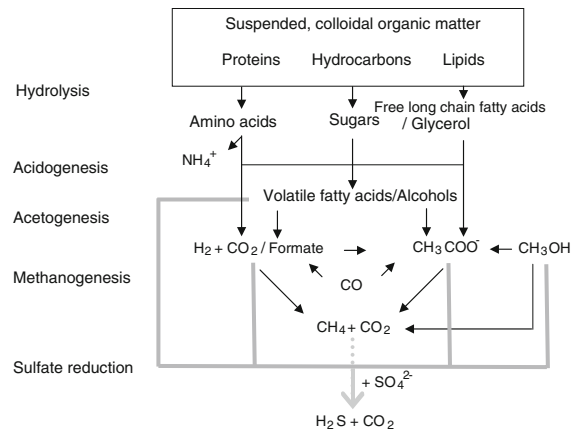
Therefore, less  $\text{CO}_2$  is produced per kWatt during the complete oxidation of  $\text{CH}_4$ .

## 1.2 Methane production

Biogas, with  $\text{CH}_4$  as the major reduced component, is produced during the biological degradation of organic matter when respiration is not possible. In the presence of inorganic electron acceptors like oxygen, nitrate, iron (III), manganese (IV) and sulfate, microorganisms oxidize organic compounds completely to  $\text{CO}_2$ . During these respiratory processes, microorganisms conserve energy for their metabolism. The reduction of oxygen is most favorable and the reduction of  $\text{CO}_2$  to  $\text{CH}_4$  is the least favorable. Sulfate reduction (SR) is only slightly more favorable than  $\text{CO}_2$  reduction. Organic matter degradation will, in general, only result in  $\text{CH}_4$  production when inorganic electron accepters are depleted.

Methanogenesis occurs in marine and freshwater sediments that are rich in organic matter, in wetlands and in the intestinal tract of insects (e.g. termites). Engineered methanogenic systems, e.g. digesters, upflow anaerobic sludge bed (UASB) and expanded granular sludge bed (EGSB) reactors, are widely applied for the treatment solid wastes and waste waters rich in organic matter. Such waste streams are produced in agriculture, households, the food and beverage industry and the paper industry (Frankin 2001). The produced biogas is recovered and can be used as fuel (Lettinga and van Haandel 1993). Anthropogenic  $\text{CH}_4$  emissions arise from agriculture and waste disposal, including enteric fermentation, animal and human wastes, rice paddies, biomass burning and landfills.

Methanogenic degradation of organic matter proceeds via a number of microbial processes; during hydrolyses, acidogenesis and acetogenesis complex organic matter is degraded to hydrogen and  $\text{CO}_2$ , formate, acetate and ammonium (Fig. 1; Harper and Pohland 1986; Stams 1994; Muyzer and Stams 2008). The final step is methanogenesis. Methanogens are strict anaerobes and belong to the archaea. Three methanogenic pathways can be distinguished: the hydrogenotrophic pathway, in which hydrogen and  $\text{CO}_2$ , formate or carbon monoxide (Daniels et al. 1977; O'Brien et al. 1984) are utilized for  $\text{CH}_4$  production; the acetoclastic pathway, in which acetate is converted to  $\text{CH}_4$  and  $\text{CO}_2$ ; and the methylotrophic



**Fig. 1** Simplified schematic representation of the anaerobic degradation process in the absence (in *black*) and in the presence (in *gray*) of sulfate

pathway, in which methanol or other methylated compounds (methanethiol, dimethyl sulfide, or methylated amines) are partly oxidized and partly converted to  $\text{CH}_4$  (Deppenmeier et al. 1996). Some methanogens are able to use pyruvate as carbon and energy source and some are able to utilize ethanol or isopropanol as electron donor for  $\text{CO}_2$  reduction (Stams 1994).

## 1.3 Sulfate reduction

Dissimilatory sulfate reduction is the reduction of sulfate to sulfide to obtain energy for growth and maintenance. This metabolic feature is exclusively done by sulfate-reducing microorganisms (SRB). SRB are a diverse group of prokaryotes (Castro et al. 2000), the known SRB can be grouped into seven phylogenetic lineages, five within the bacteria and two within the archaea (Muyzer and Stams 2008). Typically SRB occur in anoxic marine and freshwater environments (Postgate 1984). Eight electrons are needed for the reduction of one sulfate to one sulfide. The reduction equivalents are obtained by the oxidation of organic compounds or hydrogen. The different SRB are able to utilize a wide range of organic electron donors, including ethanol, formate, lactate, pyruvate, fatty acids, carbon monoxide, methanol, methanethiol and sugars (Fig. 1; Widdel et al. 2007; Muyzer and Stams 2008). SRB have a higher affinity for hydrogen than methanogens, and therefore outcompete methanogens at low hydrogen partial pressures. It has often been observed that

acetate is predominately degraded by methanogens in presence of sulfate though (van Bodegom and Stams 1999; Stams et al. 2005). Acetate-degrading sulfate reducers have only slightly better growth kinetic properties than *Methanosaeta* (dominant in anaerobic sludge). Therefore it may take years before acetate-degrading sulfate reducers are outcompeted by acetate-degrading sulfate reducers, especially when the relative cell number of the acetate-degrading sulfate reducers is initially low (Stams et al. 2005).

SR only occurs when electron acceptors with a higher redox potential (e.g. oxygen and nitrate) are absent. These sulfate-reducing conditions are found in sediments and stratified waters, in which the penetration of oxygen is limited. Sulfide produced in the anoxic compartment will be partly transported to the aerobic compartment where sulfide is oxidized to sulfate, and visa versa (Bottrell and Newton 2006; Holmer and Storkholm 2001). SR and sulfide oxidation form the main routes of the biological sulfur cycle (Fig. 2).

#### 1.4 Sources of methane in marine sediments

Seawater contains ~28 mM sulfate. Therefore organic matter oxidation in marine sediments is for a large part coupled to SR. However, when the organic matter input is large enough, sulfate will be depleted in the top part of the sediment and organic matter degradation will result in CH<sub>4</sub> production. The highest marine CH<sub>4</sub> production rates can be found near the continental margins, because the primary production in the overlying surface waters and thus also the organic matter deposition is largest in those relatively shallow waters. This CH<sub>4</sub> production by

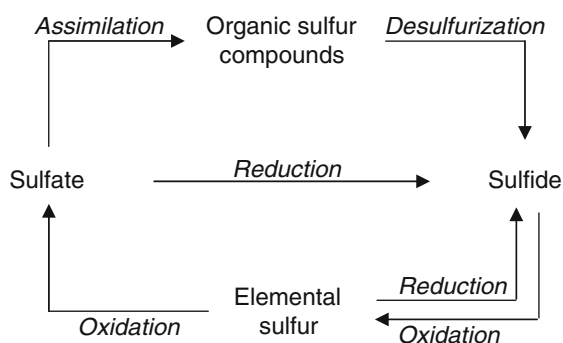
organic matter degradation is a very diffuse source for CH<sub>4</sub>.

There are also some less diffuse sites where CH<sub>4</sub> is passing up by convection along cracks and faults. These are called cold seeps or CH<sub>4</sub> vents, in which pore water or fluid with dissolved CH<sub>4</sub> seeps up from deeper sediment layers, or in which gaseous CH<sub>4</sub> vents up. This results in ecological niches with large CH<sub>4</sub> inputs. These seeps can occur in many forms, e.g. as mud volcano's (Damm and Budéus 2003; Stadnitskaia et al. 2006) or brine pools. In addition to cold seeps and vents there are hydrothermal vents where mainly CH<sub>4</sub> is being vented (Boetius 2005). These are different from the “black smokers”, in which mainly sulfide is vented.

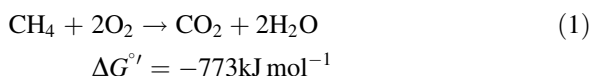
The CH<sub>4</sub> from these vents and seeps can be produced biologically, but can also be produced geochemically or thermogenically from organic matter (Sibuet and Olu 1998). CH<sub>4</sub> seeps and vents occur above fossil fuel fields or gas hydrates. Gas hydrates are ice-like structures in which a gas, mostly CH<sub>4</sub>, is incorporated. The earth's gas hydrates contain more energy than all other known oil, natural gas and coal reservoirs combined (Kvenvolden 1995). These hydrates are stable at low temperatures (<15°C), high pressures (>5.0 MPa) and in the presence of dissolved CH<sub>4</sub> (Sultan et al. 2003), but the hydrates will dissociate when they come in contact with warm fluids or when dissolved CH<sub>4</sub> is depleted (Boetius and Suess 2004).

#### 1.5 Aerobic methane oxidation

Aerobic methanotrophs are bacteria that use CH<sub>4</sub> as electron donor and carbon source (Anthony 1982; Amaral and Knowles 1995). Aerobic methanotrophs are found in samples from muds, swamps, rivers, rice paddies, oceans, ponds, soils from meadows, deciduous woods and sewage sludge (Hanson and Hanson 1996). The aerobic CH<sub>4</sub> oxidation (reaction 1) occurs via a linear pathway, in which CH<sub>4</sub> is first converted to methanol by a NADH-dependent monooxygenase. Methanol is further oxidized via formaldehyde and formate to carbon dioxide by NADH-independent methanol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase. The electrons released in these steps are passed to the electron transport chain for adenosine triphosphate (ATP) synthesis (Hanson and Hanson 1996).



**Fig. 2** The main biological processes of the sulfur-cycle

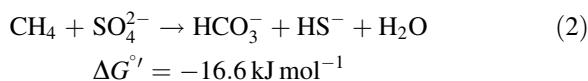


Under oxygen limiting conditions, methanotrophs can produce methanol (Xin et al. 2004; Lee et al. 2004) or acetate (Costa et al. 2000) from  $\text{CH}_4$ . Denitrifiers are able to utilize these products. In this way, denitrification with  $\text{CH}_4$  as electron donor is possible at oxygen limiting conditions (Costa et al. 2000; Waki et al. 2004). A similar process for SR has thus far not been described, although some sulfate reducers can tolerate the presence of oxygen (Muyzer and Stams 2008).

### 1.6 Anaerobic oxidation of methane

For many years anaerobic oxidation of methane (AOM) was thought to be impossible (Thauer and Shima 2008). In the 70s of the last century evidence for the occurrence of AOM was obtained during geochemical in situ studies in anaerobic marine sediments and waters.  $\text{CH}_4$  diffusing upwards from deeper sediment layers was oxidized before reaching oxic zones. The consumption of  $\text{CH}_4$  was assumed to be coupled to the consumption of sulfate, diffusing downward from the seafloor (Fig. 3; Martens and Berner 1974, 1977; Barnes and Goldberg 1976; Reeburgh 1976; Alperin and Reeburgh 1985). Radio-isotope tracer experiments with  $^{14}\text{C}$ -labeled  $\text{CH}_4$  and

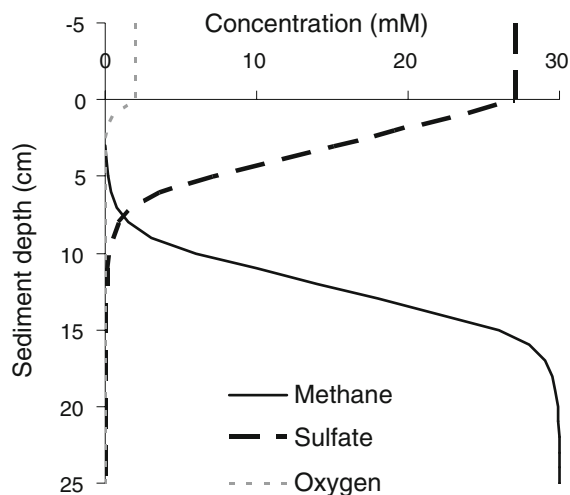
$^{35}\text{S}$ -labeled sulfate, showed a maximum AOM and SR rate at the methane sulfate transition zone (Reeburgh 1980; Iversen and Jørgensen 1985; Iversen et al. 1987; Alperin 1989; Reeburgh et al. 1991; Joye et al. 1999). In addition, at the sulfate to methane transition zone shifts in the isotopic composition ( $^{13}\text{C}$  and  $^{12}\text{C}$  content) of  $\text{CH}_4$ , which was heavier above the transition zone, and inorganic carbon, which was lighter above the transition zone, were found (Oremland and DesMarais 1983; Whiticar 1996; Oremland et al. 1987; Alperin et al. 1988; Blair and Aller 1995; Martens et al. 1999). These studies showed a stoichiometry according to reaction 2.



The bicarbonate and alkalinity production by AOM has resulted in the formation of chimney-like structures from calcium carbonate above  $\text{CH}_4$  vents (Michaelis et al. 2002; Stadnitskaia et al. 2005). These  $\text{CH}_4$  seeps or vents can also drive chemotropic ecosystems. The sulfide produced by AOM is, at least partly, transported upwards and aerobically oxidized to sulfur or sulfate, e.g. in tube worms or in microbial mats of *Beggiatoa*.

The AOM rate depends on a variety of conditions including the organic content of the sediment,  $\text{CH}_4$  supply rate, sulfate penetration in the sediment, temperature and pressure (Valentine 2002). Because of the higher supply rates, the AOM rates at  $\text{CH}_4$  seeps and vents are higher than in sediments where  $\text{CH}_4$  is just supplied by organic matter degradation (Table 1).

AOM has also been observed in non-marine environments. Iversen et al. (1987), Panganiban et al. (1979) and Eller et al. (2005) observed AOM in lakes and Grossman et al. (2002) in a landfill. In these cases AOM was probably coupled to SR. Islas-Lima et al. (2004) demonstrated for the first time denitrification with  $\text{CH}_4$  as electron donor in absence of oxygen. Raghoebarsing et al. (2006) demonstrated AOM coupled to nitrite and nitrate reduction by freshwater sediment from Twente kanaal (the Netherlands), this AOM process is mediated by bacteria via a completely other pathway than AOM coupled to SR (Ettwig et al. 2008; Thauer and Shima 2008). From AOM coupled to nitrate or nitrite reduction more energy can be conserved than from AOM



**Fig. 3** Typical  $\text{CH}_4$ , sulfate and oxygen concentration profiles in deep-sea AOM sediments where no convection takes place

**Table 1** Overview of AMO sites and rates reported in the literature

Location	Depth (m)	CH <sub>4</sub> source	AOM ( $\mu\text{mol g}_{\text{dw}}^{-1} \text{day}^{-1}$ )		References
			Radiotracers	In vitro	
Eckernförde Bay, Baltic Sea	28	Organic matter decomposition	0.03–0.06	0.1–0.3	Treude et al. (2005a)
Kattegat, Baltic Sea	0.5	Organic matter decomposition	0.05–0.2	0.05–1	Krüger et al. (2005)
Spiekeroog, North Sea	0–5	Organic matter decomposition	ND	0.01–0.2	Krüger et al. (2005)
Aarhus Bay, Denmark	16	Organic matter decomposition	ND	ND	Thomsen et al. (2001)
Black Sea	250	Fossil methane	0.2–7.5 8–21	0.5–3.5	Krüger et al. (2005) and Treude et al. (2007)
Haakon Mosby Mud Volcano, Atlantic Ocean	1,250	Fossil methane	ND	0.1–1	Damm and Budéus (2003)
Gulf of Cadiz, Atlantic Ocean	400–3,000	Mud Volcano	ND	ND	Niemann et al. (2006) and Stadnitskaia et al. (2006)
Namibiaan margin, Atlantic Ocean	25	Organic matter decomposition	ND	ND	Niewöhner et al. 1998
Gulf of Mexico	650	Gas hydrates	ND	1–13	Joye et al. (2004) and Krüger et al. (2005)
Hydrate Ridge, Pacific Ocean	700	Gas hydrates	0.3–6	2–8	Boetius et al. 2000, Treude et al. (2003) and Krüger et al. (2005)
Monterey Bay, Pacific Ocean	800–1,000	Cold seep	ND	0.03	Girguis et al. (2003, 2005)
Eel River Basin, Pacific Ocean	516–556	Gas hydrates	ND	ND	Orphan et al. (2002)
Chilean margin, Pacific Ocean	800–4,600	Organic matter decomposition	0.001–0.07	ND	Treude et al. (2005b)
Pearl River estuary, Pacific Ocean	3–4	Organic matter decomposition	ND	ND	Wu et al. (2006)

ND not determined

coupled SR. The same would be true for AOM coupled to iron (III) or manganese (IV) reduction. Thus far there is no direct evidence for AOM coupled to iron (III) or manganese (IV) reduction; however, Beal et al. (2009) did demonstrate an iron and manganese dependency of methane oxidation in marine sediments.

### 1.7 Relevance of the anaerobic oxidation of methane for global warming

Estimates of the current human-activity-related CH<sub>4</sub> emissions range from 340 to 420 Tg CH<sub>4</sub> year<sup>-1</sup>, while the total natural terrestrial sources are estimated to be between 160 and 270 Tg CH<sub>4</sub> year<sup>-1</sup> (Khalil and Shearer 2000; Lelieveld et al. 1998; Houweling et al. 1999). The annually CH<sub>4</sub> production in anoxic marine sediments is probably more than 85 Tg (Hinrichs and

Boetius 2002). CH<sub>4</sub> is after CO<sub>2</sub> the most important greenhouse gas, responsible for 20% of the infrared radiation trapping in the atmosphere (Mackenzie 1998). The lifetime of CH<sub>4</sub> in the atmosphere is shorter than that of CO<sub>2</sub>, but the strong global warming effect is due to the fact that a relative high fraction of the CH<sub>4</sub> occurs in the troposphere. Atmospheric CH<sub>4</sub> is mainly oxidized in the troposphere, by the reaction with a hydroxyl radical (OH·), this accounts for a removal of 445–530 Tg CH<sub>4</sub> per year. Just 40 Tg CH<sub>4</sub> year<sup>-1</sup> is transported to the stratosphere. In aerated soils, about 30 Tg CH<sub>4</sub> is annually oxidized by aerobic methanotrophs (Khalil and Shearer 2000; Lelieveld et al. 1998; Houweling et al. 1999). Initial AOM was estimated to be responsible for 75 Tg CH<sub>4</sub> removal per year (Reeburgh 1996). Later estimates suggested that 300 Tg CH<sub>4</sub> was annually removed by AOM (Hinrichs and Boetius 2002), which would make AOM the

second most important process for removal of the greenhouse gas  $\text{CH}_4$ .

## 2 Sulfate reduction in biotechnology

### 2.1 Environmental problems related with the sulfur cycle

Sulfur compounds are cycled between the earth's soils, oceans, atmosphere and living matter in the so-called "natural sulfur cycle". However, due to human activities the emissions of sulfur compounds to surface waters and the atmosphere have increased largely. The earth's crust contains large amounts of immobilized sulfides. During mining and processing of ores and fossil fuels, sulfide minerals are oxidized and have been emitted to the surface waters, soils and the atmosphere. This has caused major environmental problems like the acidification of surface waters, the mobilization of toxic metals, the increasing salinity of freshwaters and the production of toxic sulfide in anaerobic soils (Morin et al. 2006).

Here three important sources of anthropogenic sulfur emissions are distinguished. The first are waste streams of the mining and metallurgical industry. During the mining of metal ores, minerals like pyrite are biologically oxidized (Johnson 2000), resulting in the production of sulfuric acid and the mobilization of metals. Many metals are toxic for humans and have a devastating effect on ecosystems. This mining wastewater is called acid mine drainage. During the processing of these minerals at metallurgical plants, waste streams with sulfuric acid, sulfur dioxide and residual metals are also produced. The second source of sulfurous emissions is the combustion of fossil fuels. Fossil fuels (like coal, oil and gas) contain S-compounds. Their combustion results in the emission of sulfur dioxide, a major compound in the acid rain formation. Therefore, sulfur dioxide has to be removed from the off-gas (flue gas desulfurization) or sulfur compounds have to be removed from fuels prior to combustion, both processes result in the generation of a waste stream containing the sulfur compounds. A third source are wastewaters contaminated with oxidized sulfur compounds (sulfate, sulfite and thiosulfate) that are produced in industries that use sulfuric acid or sulfate-rich feedstock, e.g. tannery, pulp and paper, textiles, fermentation and the

sea food processing industry (Lens et al. 1998). Annually 136 Tg sulfuric acid is used in the industry (Kirk-Othmer 2000).

### 2.2 Removal and recovery of metals and oxidized sulfur compounds

SR in anaerobic bioreactors treating organic wastes has long been regarded as an unwanted side process due to the loss of electron donor and inhibition of the methanogenic process by sulfide (Colleran et al. 1995; Oude Elferink et al. 1994). Currently, biological SR is an established biotechnological process for the treatment of inorganic waste streams containing sulfur compounds and/or metals (Weijma et al. 2002; Lens et al. 2002). Oxidized sulfur compounds can be converted to elemental sulfur by applying subsequently SR and partial sulfide oxidation (Janssen et al. 1999; van den Bosch 2008). The insoluble sulfur can be recovered by means of a settler and is a safe, storable and reusable product. The hydrophilic nature of biologically produced sulfur makes it an ideal soil fertilizer, in addition, sulfur can be used to produce sulfuric acid (van den Bosch 2008). Most cationic metals, e.g.  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$ , can be removed from the solution by precipitation with biologically produced sulfide, the formed insoluble metal sulfides can be separated from the water phase in a settler and reused in the metallurgical industry (Huisman et al. 2006; Veeken et al. 2003). These biological treatment techniques allow the recovery of sulfur and metals; they can be used for the treatment of acid mine drainage, groundwater leachate, industrial wastewaters and industrial waste gases (containing  $\text{SO}_2$  or  $\text{H}_2\text{S}$ ). In addition, SR can be applied in situ, in order to immobilize metals as metal sulfides in soils and sediments.

Biological SR forms a relative new alternative to remove sulfate from liquid streams for the widely applied chemical precipitation, in which sodium sulfate or gypsum is produced. Gypsum can be reused as construction material. However, the sulfate containing waste streams from the mining and metallurgical industry are polluted with metals, the produced gypsum will therefore be polluted as well and needs to be stored as chemical waste. For chemical precipitation, large amounts of chemicals are needed, per kg sulfate about 0.8 kg slaked lime is needed. During slaked lime production from limestone  $\text{CO}_2$  is



released, additional to the CO<sub>2</sub> produced related to the energy consumption of the process (the process requires a temperature of 900°C). Because of a lower CO<sub>2</sub> emission and the production of a reusable product, biological treatment of wastewaters containing sulfate and metals is more sustainable than treatment by chemical precipitation.

### 2.3 Electron donors for sulfate reduction

The costs of the electron donor forms a major part of the running cost of a SR process and therefore limit the application of biological SR as it cannot always economically compete with chemical precipitation. Cheap electron donors like organic waste streams are not easily degradable and often contain some inert material, which would need to be removed by pre or post treatment. In addition, undesired byproducts can be formed and the quantity and quality of these waste streams is not constant. Easily degradable bulk chemicals are therefore a better option. Such electron donors include hydrogen, synthesis gas, methanol, ethanol, acetate, lactate, propionate, butyrate, sugar, and molasses (Liamleam and Annachhatre 2007),

many of which have been extensively investigated as electron donor for SR in bioreactors (Table 2). According to van Houten (1996) hydrogen is the best electron donor at large scale (>5–10 kmol SO<sub>4</sub><sup>2-</sup> h<sup>-1</sup>), while ethanol is an interesting electron donor at smaller and middle scale.

#### 2.3.1 Hydrogen

Two advantages of gaseous electron donors are that the wastewater is not diluted and that the electron donor can not wash-out with the effluent. A disadvantage of gaseous electron donors is that they are voluminous and therefore need to be compressed during transportation. High rate SR with H<sub>2</sub> as electron donor and carbon dioxide (CO<sub>2</sub>) as carbon source has been demonstrated at both mesophilic and thermophilic conditions (Table 2). A maximum SR rate of 30 g SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> day<sup>-1</sup> was reached. Van Houten (2006) showed that in a H<sub>2</sub> and CO<sub>2</sub> fed gas-lift bioreactor, SRB do not take CO<sub>2</sub> as sole carbon source, instead they depend on the acetate produced by homoacetogens. Hydrogenotrophic methanogens compete with SRB for the available H<sub>2</sub>, using CO<sub>2</sub> as

**Table 2** Effect of electron donor, pH, temperature and reactor concept on the volumetric sulfate-reducing activity

e-donor	pH	Temp (°C)	Reactor concept	Volumetric activity (gSO <sub>4</sub> <sup>2-</sup> L <sup>-1</sup> day <sup>-1</sup> )	Reference
Hydrogen	8.0	30	GLB	25	van Houten et al. (2006)
Hydrogen	7.0	30	GLB	30	van Houten et al. (1994)
Hydrogen	7.0	55	GLB	8	van Houten et al. (1997)
Hydrogen	6.0	30	GLB	13	van Houten et al. (1995a)
Synthesis gas(80% H <sub>2</sub> and 20% CO)	7.0	30	GLB	7	van Houten et al. (1995b)
Synthesis gas	— <sup>a</sup>	35	Anaerobic packet bed reactor	1.2	du Preez and Maree (1994)
CO	— <sup>a</sup>	35	Anaerobic packet bed reactor	2.4	du Preez and Maree (1994)
CO	6.9	50–55	GLB	0.2	Sipma et al. (2007)
Formate	6.0	30	MBR	29	Bijmans et al. (2008)
Methanol	7.5	65	EGSB	15	Weijma et al. (2000)
Ethanol	8	35	FBR	5	Kaksonen et al. (2004)
Ethanol	7	8	FBR	0.6	Sahinkaya et al. (2007)
Ethanol	7.2	33	MBR	0.6	Vallero et al. (2005)
Acetate	8	35	Fixed bed bioreactor	65	Stucki et al. (1993)
Acetate	8	33	EGSB	10	Dries et al. (1998)

<sup>a</sup> Not controlled

terminal electron acceptor. In a well-mixed stable-performing bioreactor, the consortium of heterotrophic SRB and homoacetogens outcompetes methanogens, because of a higher affinity for  $H_2$ . At elevated  $H_2$  concentrations (e.g. during startup, in poorly mixed systems or after a disturbance) methanogens are able to grow, resulting in a loss of electron donor due to methanogenesis (van Houten et al. 2006).

Hydrogen is commonly produced by steam reforming from natural gas or by gasification of oil or coal (Armor 1999; Bartish and Drissel 1978). Steam reforming takes place at high temperatures (750–800°C) and pressures (0.3–2.5 MPa) in the presence of a nickel-based catalyst, the efficiency ranges from 60 to 80%. The gas produced by steam reforming or gasification (synthesis gas) contains, besides hydrogen, between 6 and 60% carbon monoxide (CO; Bartish and Drissel 1978). CO can be removed via the so called water–gas-shift reaction, in which CO and water react over a chemical catalyst at 360°C to form carbon dioxide and hydrogen. To limit methanogenic and homoacetogenic activity the carbon dioxide can subsequently be removed from the gas (e.g. using an alkaline scrubber). More sustainable ways to produce hydrogen are emerging, e.g. gasification of organic waste or biomass (van der Drift et al. 2001), electrolysis using “green” electricity, hydrogenogenic phototrophic microorganisms (Hoekema et al. 2002), dark fermentation (Nath and Das 2004) and biocatalyzed electrolyses in a fuel cell (Rozendal et al. 2006).

### 2.3.2 Synthesis gas

The chemical water–gas-shift reaction has two disadvantages. Firstly, the chemical catalysts become polluted by hydrogen sulfide which is also present in synthesis gas and secondly, the chemical process

requires a high temperature and pressure. Alternatively the untreated synthesis gas, including the CO, could be fed to the SR bioreactor. Van Houten (1995b) found that the SR rate dropped from 12 to 14 g  $SO_4^{2-} L^{-1} day^{-1}$  to 6–8 g  $SO_4^{2-} L^{-1} day^{-1}$  when adding 5% CO to the  $H_2/CO_2$  feed gas. Increasing the percentage CO to 20% did not further deteriorate the SR rate. However, Sipma et al. (2004) showed that some SRB were able to tolerate up to 100% CO. At thermophilic conditions, the responsible microorganisms could convert CO and  $H_2O$  to  $H_2$  and  $CO_2$  and simultaneously use the  $H_2$  for SR. Although CO is inhibitory for methanogenesis, methanogens could only be eliminated at a short hydraulic retention time (3 h) in a synthesis gas fed gas-lift bioreactor (Sipma et al. 2007).

### 2.3.3 Methane

Another alternative would be the use of natural gas or biogas directly as electron donor for biological SR. This would have four advantages. Firstly, the steam reforming and the carbon monoxide removal are avoided. These processes contribute to the additional costs of hydrogen over  $CH_4$ . The costs for the electron donor would be reduced by factor 4 if natural gas instead of hydrogen or ethanol was used as electron donor (Table 3). Secondly, the chemical catalysts used for steam reforming and the water–gas shift are easily polluted by hydrogen sulfide, present in the natural gas or biogas. Sulfide forms no problem when the  $CH_4$  containing gas would be fed directly to the bioreactor. Thirdly, energy needed for the transfer of the gas to the liquid can be saved. Four times less gas needs to be transferred from the gas to the liquid phase, as one  $CH_4$  can donate eight electrons, and one hydrogen only two. In addition, the solubility of  $CH_4$

**Table 3** Prices and costs of electron donors for sulfate reduction

Electron donor	Industrial market price	Required amount per kg sulfate reduced	Electron donor cost [ $\$ kg_{sulfate}^{-1}$ ]
Ethanol	0.65 $\$ L^{-1}$ <sup>a</sup>	0.40 L	0.26
Hydrogen	0.21 $\$ m^{-3}$ <sup>b</sup>	0.934 $m^3$	0.20
Natural gas	0.16 $\$ m^{-3}$ <sup>c</sup>	0.292 $m^3$	0.05

<sup>a</sup> Ethanol Market, <http://ethanolmarket.aghost.net/>, accessed December 2009

<sup>b</sup> Mueller-Langer et al. (2007)

<sup>c</sup> Energy Information Administration, [http://tonto.eia.doe.gov/dnav/ng/ng\\_pri\\_sum\\_dcu\\_nus\\_m.htm](http://tonto.eia.doe.gov/dnav/ng/ng_pri_sum_dcu_nus_m.htm), accessed December 2009



(1.44 mM in distilled water at 0.101 MPa CH<sub>4</sub> and 20°C) is higher than of hydrogen (0.817 mM at 0.101 MPa hydrogen and 20°C). The volumetric conversion rates in bioreactors fed with a gaseous substrate are, in general, limited by the transfer of the gas to the liquid phase.

A third advantage is that substrate losses due to unwanted methanogenesis and acetogenesis (from hydrogen and CO<sub>2</sub>) can be avoided, only microorganisms involved in AOM coupled to SR are able to grow in a CH<sub>4</sub>-fed sulfate-reducing bioreactor.

## 2.4 Reactor type

The gas-lift bioreactor (GLB) is the most common bioreactor type for SR with gaseous electron donors. In this system the transfer of gas to the liquid is optimized. A GLB is usually equipped with a three-phase separator (Esposito et al. 2003; van Houten et al. 1994; Weijma et al. 2002) or an external settler (Sipma et al. 2007) to retain the biomass in the system. GLBs can be operated with (van Houten et al. 1994) or without (Sipma et al. 2007) carrier material like pumice and basalt. Metal-sulfides produced in gas-lift bioreactors can also act as carrier material for the microorganisms.

Membrane bioreactors (MBRs) are relatively new in the field of SR. The advantage is that almost complete biomass retention can be obtained, which is especially useful when slow-growing microorganisms are used. MBRs have been applied in research on SR under high saline conditions (Vallero et al. 2005) and SR at low pH (Bijmans et al. 2008).

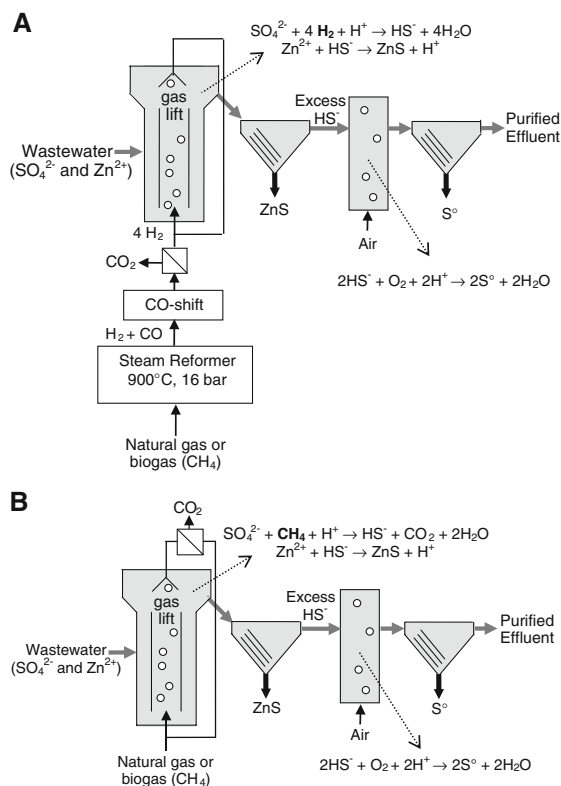
## 2.5 The wastewater treatment process at Nyrstar

At the Nyrstar zinc refinery in Budel (the Netherlands), SR is applied to separate and recover sulfuric acid and zinc from waste streams that also contain other dissolved compounds, e.g. Mg<sup>2+</sup> and Cl<sup>−</sup>. The waste streams are treated in a single-stage hydrogen-fed 500 m<sup>3</sup> GLB. In the GLB, SR and zinc-sulfide precipitation take place (Boonstra et al. 1999; Weijma et al. 2002). The sulfate concentration is reduced from 5–15 to 0.05 g L<sup>−1</sup>, while the zinc concentration is reduced to less than 0.3 mg L<sup>−1</sup>, recovering about 8.5 tons of zinc-sulfide per day (Boonstra et al. 1999; Weijma et al. 2002). The recovered zinc-sulfide can be directly reused in the

zinc smelter. At the Nyrstar zinc refinery, hydrogen produced by steam CH<sub>4</sub> reforming is used as electron donor for biological SR. The relative small steam

**Table 4** Basic parameters of the current wastewater treatment process at the zinc refinery of Nyrstar (Budel, the Netherlands) and of the wastewater treatment process when CH<sub>4</sub> would be used directly as electron donor for biological SR

	SR with CH <sub>4</sub> via H <sub>2</sub> production plant	SR with CH <sub>4</sub> directly
	Three step process	One step process
Temperature required	900°C	Wastewater temperature (5–70°C)
Pressure required	1.6 Mpa (16 bar)	0.1 Mpa (1 bar)
CH <sub>4</sub> required	1.88 mol per mol SO <sub>4</sub> <sup>2−</sup>	1 mol per mol SO <sub>4</sub> <sup>2−</sup>
CO <sub>2</sub> emission	0.9 ton per ton SO <sub>4</sub> <sup>2−</sup>	0.45 ton per ton SO <sub>4</sub> <sup>2−</sup>



**Fig. 4** Simplified schematic representation of the current wastewater treatment process at the zinc factory of Nyrstar in Budel (the Netherlands; **a**). The wastewater treatment process when CH<sub>4</sub> would be used as direct electron donor (**b**)

reformer needs 1.88 mol  $\text{CH}_4$  to reduce 1 mol sulfate.

Table 4 compares the current SR process at Nyrstar (Fig. 4a) with the theoretical process if  $\text{CH}_4$  would be used as electron donor for biological SR (Fig. 4b). From the stoichiometry of AOM coupled to SR, a consumption of one mol  $\text{CH}_4$  per mol sulfate can be expected. Because less  $\text{CH}_4$  is needed and less energy is needed for gas recirculation, the carbon dioxide emission of the process in which  $\text{CH}_4$  is used directly is expected to be half of the current  $\text{CO}_2$  emission.

### 3 Microbial aspects of sulfate reduction with methane as electron donor

#### 3.1 Anaerobic methanotrophs

In contrast to aerobic  $\text{CH}_4$  oxidation, the biochemistry of AOM coupled to SR is not completely understood. AOM is mediated by uncultured Archaea, called anaerobic methanotrophs (ANME). Specific archaeal lipids (biomarkers), from in situ samples, are highly depleted in  $^{13}\text{C}$  (Elvert et al. 1999, 2001; Hinrichs et al. 1999, 2000; Thiel et al. 1999, 2001; Pancost et al. 2000). This is evidence that the isotopically light  $\text{CH}_4$  (biologically produced  $\text{CH}_4$  is depleted in  $^{13}\text{C}$ ) was the preferred carbon source for these microorganisms rather than other “heavier” carbon sources. Phylogenetic analysis of AOM sediments identified three novel groups of archaea, called ANME-1, ANME-2 and ANME-3. ANME-1 and ANME-2 are most abundant and geographically widespread. ANME are phylogenetically distantly related to cultivated methanogenic members from the orders *Methanosarcinales* and *Methanomicrobiales* (Hinrichs et al. 1999; Orphan et al. 2002; Knittel et al. 2005; Niemann et al. 2006). Orphan et al. (2001a, 2002) combined isotopic and phylogenetic analysis and showed that cells belonging to ANME-1 and ANME-2 assimilated carbon from  $\text{CH}_4$  during AOM.

#### 3.2 Reversed methanogenesis

AOM is a form of reversed methanogenesis: AOM is like methanogenesis inhibited by bromoethanesulfonate (BES; Nauhaus et al. 2005), ANME-1 cells were

found to contain most of the genes typically associated with  $\text{CH}_4$  production (Hallam et al. 2003, 2004) and an analogue of the methyl-coenzyme M reductase was found to make up 7% of the extracted soluble proteins from an AOM mediating microbial mat from the Black Sea (Krüger et al. 2003). The  $\Delta G^\circ$  of the reduction of methyl-coenzyme M to produce  $\text{CH}_4$  is  $-30 (\pm 10) \text{ kJ mol}^{-1}$ , the back reaction becomes exogenic when the product to substrate concentration ratio is  $\sim 10^5$ , such a ratio is physiologically not unrealistic (Thauer and Shima 2008). In addition, pure cultures of methanogenic archaea and methanogenic mixed cultures also oxidize  $\text{CH}_4$  to  $\text{CO}_2$  in the absence of oxygen, but in low amounts and during net methanogenesis (Zehnder and Brock 1979; Harder 1997; Moran et al. 2004; Moran et al. 2007; Meulepas et al. 2010). SRB did not show any  $\text{CH}_4$  oxidation during SR (Harder 1997).

Thus far, there is no direct evidence that ANME are capable of methanogenesis. However, AOM and  $\text{CH}_4$  production occur simultaneously in microbial mats from the Black Sea (Seifert et al. 2006), in sediments from Cape Lookout Bight (North Carolina; Hoehler et al. 1994) and in sediments from the Gulf of Mexico (Orcutt et al. 2005).  $\text{CH}_4$  production by Hydrate Ridge sediment on hydrogen, formate, acetate and methanol, in absence of  $\text{CH}_4$ , was an order of a magnitude lower than the AOM rate though (Nauhaus et al. 2002), and microbial mats from the Black Sea did not show any  $\text{CH}_4$  production in presence of hydrogen and absence of sulfate (Treude et al. 2007). In addition, growth of ANME on solely methanogenic substrates has not been reported.

#### 3.3 SRB associated with AOM

Some archaea (belonging to the Euryarchaeota or Crenarchaeota) are capable of SR (Muyzer and Stams 2008). However, in the archaea belonging to the ANME groups, no gene analogues for enzymes involved in SR were found (Thauer and Shima 2008). In addition, methyl-coenzyme M reductase was shown to be inhibited by sulfite, an intercellular intermediate of SR (Mahlert et al. 2002). Therefore, it is unlikely that AOM and SR take place in the same cell (Shima and Thauer 2005). At AOM sites, ANME co-occur with SRB belonging taxonomically to the delta group of proteobacteria and associated with the

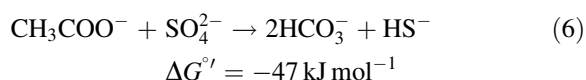
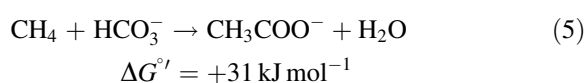
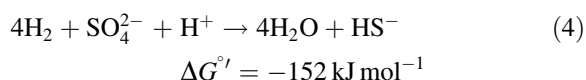
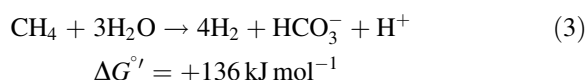
*Desulfosarcina/Desulfococcus* cluster (Boetius et al. 2000; Orphan et al. 2001b; Michaelis et al. 2002; Elvert et al. 2003; Knittel et al. 2003). During incubations of AOM sediment with  $^{13}\text{C}$ -labeled  $\text{CH}_4$ ,  $^{13}\text{C}$  was incorporated both in archaeal lipids associated with ANME and bacterial lipids associated with SRB. This incorporation in bacterial lipids might proceed via a carbon compound produced from  $\text{CH}_4$  by ANME rather than by the direct uptake of  $\text{CH}_4$  by SRB (Blumenberg et al. 2005). It has frequently been suggested that an archaeon produces an electron carrier compound from  $\text{CH}_4$  that is utilized by a sulfate-reducing partner (Fig. 5; Zehnder and Brock 1980; Alperin and Reeburgh 1985; Hoehler et al. 1994 and DeLong 2000). In sediment from Hydrate Ridge, Eel River Basin and the Gulf of Mexico, ANME-2 and SRB live in consortia with a diameter of up to circa 20  $\mu\text{m}$  (Boetius et al. 2000; Hinrichs et al. 2000; Knittel et al. 2005). Moreover, both microorganisms were growing in consortia with  $\text{CH}_4$  and sulfate as sole substrates (Nauhaus et al. 2007), confirming the involvement of the SRB in AOM coupled to SR.

These ANME/SRB aggregates are not dominant in all AOM sites though. In Black sea microbial mats, SRB mainly occur in microcolonies surrounded by bulk ANME-1 cells clusters (Michaelis et al. 2002; Knittel et al. 2005). The distances between ANME and SRB in those microbial mats are larger than in the consortia from Hydrate Ridge. In samples from Eel River Basin ANME-1 archaeal group frequently

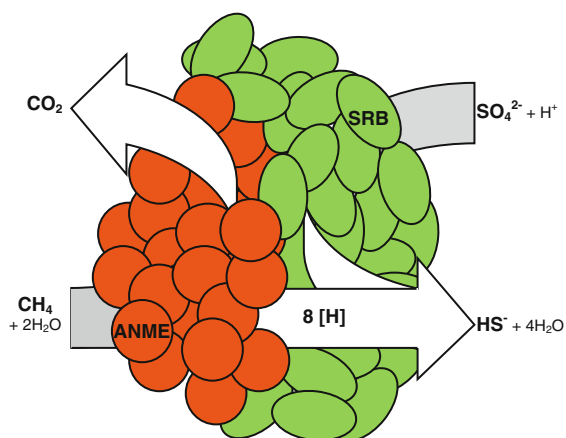
existed in monospecific aggregates or as single filaments, apparently without a bacterial partner (Orphan et al. 2002). In Eckernförde Bay sediment and in an Eckernförde Bay enrichment, clusters of ANME-2 cells were found without sulfate-reducing partners (Treude et al. 2005a; Jagersma et al. 2009).

### 3.4 Possible syntrophic routes

Given the evidence for reversed methanogenesis, hydrogen (reactions 3 and 4) and acetate (reactions 5 and 6) were initially proposed to act as interspecies electron carrier (IEC; Hoehler et al. 1994; DeLong 2000). The standard Gibbs free energy change at pH 7 ( $\Delta G^\circ'$ ) of the production of these IECs from  $\text{CH}_4$  is positive, however, when the IEC concentration is kept low enough by the sulfate-reducing partner, the  $\Delta G$  will be negative.



There are some thermodynamic concerns about this theory. At in situ conditions there is only  $-22 \text{ kJ mol}^{-1}$  available for AOM coupled to SR (Harder 1997). This energy would need to be shared between the syntrophic partners. Methanogenic archaea have been shown to require a free energy change of at least  $-10 \text{ kJ mol}^{-1}$  and SRB of at least  $-19 \text{ kJ mol}^{-1}$  to support their metabolism in situ (Hoehler et al. 2001; Dale et al. 2006). The in situ free energy change is therefore probably not sufficiently large to fuel the energy metabolism of two microorganisms (Schink 1997; Thauer and Shima 2008). Moreover, for diffusive transport between the syntrophic partners a concentration gradient is needed. Therefore, the IEC concentration near the SRB will be lower than the concentration near the ANME and the actual available energy for the microorganisms will be even lower. The bigger the distance between the syntrophic partners the greater



**Fig. 5** Schematic representation of the proposed interspecies electron transfer in consortia composed of methane oxidizing archaea (left) and sulfate reduction bacteria (right)

the loss (Sørensen et al. 2001). Thermodynamic calculations excluded hydrogen, acetate and methanol as IEC, because the maximum diffusion distances of those compounds at in situ concentrations and rates were smaller than the thickness of two prokaryotic cell walls (Sørensen et al. 2001). Also activity assays provided evidence against potential IECs. SR activity of Hydrate Ridge sediment with hydrogen, formate or acetate was lower than SR activity on  $\text{CH}_4$ , indicating that SRB involved in AOM, were not adapted to these substrates (Nauhaus et al. 2002, 2005). Moreover, Meulepas (2009) excluded hydrogen, formate, acetate methanol and carbon monoxide as IEC's in AOM by an ANME-2 enrichment. It therefore remains unclear if and how reducing equivalents are transferred from the ANME to a sulfate-reducing partner.

#### 4 Biotechnological aspects of sulfate reduction with methane as electron donor

SR coupled to AOM has thus far mostly been studied to get a better understanding of carbon and sulfur cycling in nature. However, recent physiological in vitro and bioreactor studies provided insights in the potential of sulfate reduction with methane as electron donor for applications, and the operational window of such process.

##### 4.1 Effect of temperature, pH and salinity

The SR rates of Hydrate Ridge sediment, Black Sea microbial mats, Eckernförde Bay sediment and Eckernförde Bay enrichment were highest between 5 and 16°C (Nauhaus et al. 2005), 16 and 24°C (Nauhaus et al. 2005), 20 and 28°C (Treude et al. 2005a), and 15 and 25°C (Meulepas et al. 2009b), respectively. For biotechnological applications, the low temperature optima form a limitation, as many industrial wastewaters are warmer than 20°C. However, in many countries legislation requires treated wastewater to be cooled before discharge. Moreover, if the wastewater is cooled in a heat exchanger the energy loss can be minimized.

Many sulfate and metal containing wastewaters are acid (Weijma et al. 2002; Kaksonen and Puhakka 2007). AOM coupled to SR has thus far not been demonstrated at acid conditions; the  $\text{CH}_4$  oxidation

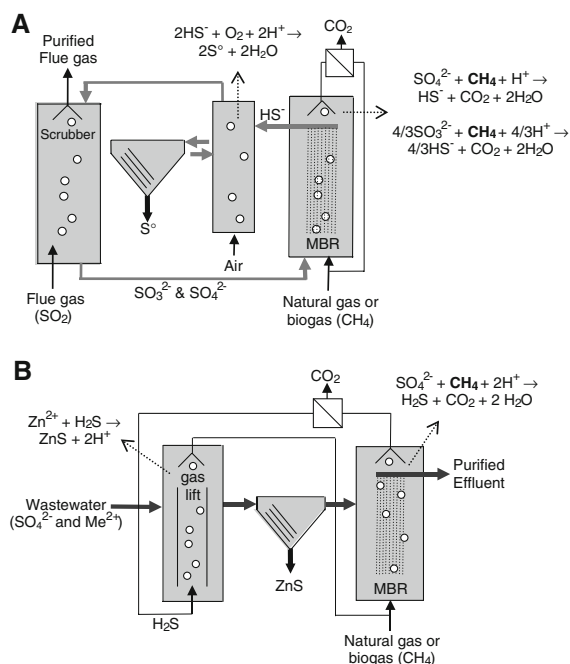
and sulfate reduction rates of an Eckernförde Bay enrichment were the highest at a pH of 7.5 and a salinity of 30‰ (Meulepas et al. 2009b), which are common optima for marine microorganisms. However, below a pH of 6.5,  $\text{H}_2\text{S}$  and  $\text{CO}_2$  will be the main products of sulfate reduction, instead of  $\text{HS}^-$  and  $\text{HCO}_3^-$ . This will result in the generation of alkalinity. Therefore, a sulfate-reducing bioreactor fed with acidic wastewater, can often be maintained at a neutral pH. The high salinity requirement makes that wastewaters low in salts (other than sulfate) cannot be treated with the AOM biomass from marine sediments. However, for applications in which the liquid is recirculated (e.g. flue gas desulfurization; Lens et al. 2003), a high salinity optimum is even an advantage, since salts accumulate in such treatment systems. Figure 6a shows a flue gas desulfurization process in which methane is used as electron donor.

##### 4.2 Effect of substrate and product concentrations

There is a positive relation between the conversion rate and the  $\text{CH}_4$  partial pressure in  $\text{CH}_4$ -oxidizing sulfate-reducing sediments (Krüger et al. 2005; Nauhaus et al. 2005) and enrichments (Meulepas et al. 2009b), even up to a pressure of 45 MPa (Kallmeyer and Boetius 2004). This implies that at ambient pressure sulfate reduction with methane as electron donor is always limited by the  $\text{CH}_4$  partial pressure. This could be overcome by applying elevated  $\text{CH}_4$  partial pressures. However, the energy required to pressurize  $\text{CH}_4$  and the additional safety hazards make the use of high-pressure bioreactor at full-scale less appealing. For ambient-pressure applications, it would be advisable to optimize the availability of  $\text{CH}_4$  for the microorganisms by applying thorough mixing,  $\text{CH}_4$  gas sparging and gas recirculation.

The ability of a  $\text{CH}_4$ -oxidizing sulfate-reducing Eckernförde Bay enrichment to remove sulfate almost completely (down to 0.05 mM; Meulepas et al. 2009b), makes it possible to use this process for sulfate removal.

Sulfide is toxic for all sulfate-reducing bacteria and methanogenic archaea. The toxicity of sulfide is often associated with its undissociated form ( $\text{H}_2\text{S}$ ) due to the facilitated passage of neutral molecules across cell membranes and to its reactivity with cellular compounds (O'Flaherty et al. 1998).



**Fig. 6** Schematic representation of a biological zinc removal process, in which sulfate reduction and metal precipitation are separated in order to prevent a loss in methanotrophic sulfate-reduction biomass (a). Schematic representation of biological flue gas desulfurization with methane as electron donor (b)

However, the sulfide tolerance of different OAM communities seem to vary; sulfide accumulated to maximum 2.4 mM (Meulepas et al. 2009b), 10 mM (Joye et al. 2004), 14 mM (Nauhaus et al. 2005) and 15 mM (Valentine 2002) in CH<sub>4</sub>-oxidizing sulfate-reducing sediments.

#### 4.3 Alternative electron acceptors

Sediments or enrichments mediating sulfate reduction with methane as electron donor, were not able to utilize nitrate (Meulepas et al. 2009b), fumarate, iron(III) or Mn(IV; Nauhaus et al. 2005) as alternative electron acceptor for methane oxidation, but were able to use thiosulfate and sulfite (Meulepas et al. 2009b). These alternative electron acceptors have application possibilities as well. Thiosulfate containing wastewater is produced at pulp bleaching and by the photographs fixing process (Lens et al. 1998), and sulfite is the main compound in the liquid from flue gas scrubbing.

#### 4.4 Growth in bioreactors

Estimates of the doubling time of the microorganisms mediating AOM coupled to SR vary from 1 to 7 months (Girguis et al. 2005; Nauhaus et al. 2007; Krüger et al. 2008; Meulepas et al. 2009a). Because of this low growth rate, biomass retention is crucial for applications of the process. Meulepas et al. (2009a) showed that CH<sub>4</sub>-oxidizing sulfate-reducing biomass could be grown in an ambient-pressure MBR. A MBR allows complete cell retention, but requires energy input to overcome the trans-membrane pressure and to prevent clogging. Thus far, it is unknown whether sufficient CH<sub>4</sub>-oxidizing sulfate-reducing biomass can be retained in a bioreactor by settling alone, like in gas-lift bioreactors or UASB systems. Although the turbulent conditions encountered in a MBR did not seem to be a problem for CH<sub>4</sub>-oxidizing sulfate-reducing mixed-cultures (Meulepas et al. 2009a), the formation of CH<sub>4</sub>-oxidizing sulfate-reducing biofilms under turbulent reactor conditions has not yet been described. Naturally AOM mediating biofilms do occur though, in the form of microbial mats in the Black Sea (Michaelis et al. 2002).

From the growth rate ( $\mu$ ) and the specific conversion rate ( $V$ ), the growth yield ( $Y$ ) can be calculated according to the formula  $Y = \mu V^{-1}$ . Nauhaus et al. (2007) calculated a molar yield of 0.6 g cell dry weight (mol CH<sub>4</sub> oxidized)<sup>-1</sup>. This was based on the sulfate reduction rate per gram ANME/SRB consortia. The low growth yield makes it difficult to combine AOM coupled to SR and metal precipitation in one system, since the metal sulfides need to be harvested without a loss of biomass. However, sulfate reduction with CH<sub>4</sub> as electron donor can be used to remove and recover metals from wastewater if SR and metal precipitation are separated, like illustrated in Fig. 6b.

### 5 Recommendations for further research

The low growth rate of the microorganisms mediating AOM coupled to SR forms a major bottleneck for biotechnological applications. The thus far highest AOM and SR rate obtained in a bioreactor is 1.0 mmol g<sub>VSS</sub><sup>-1</sup> day<sup>-1</sup> (Meulepas et al. 2009a). The full-scale sulfate-reducing bioreactor at Nyrstar (Budel, the Netherlands) is capable of reducing 87.5 kmol (8.4 ton) sulfate per day (Weijma et al. 2002).



At a doubling time of 3.8 months (Meulepas et al. 2009a), it would take 8.6 years to grow enough CH<sub>4</sub>-oxidizing sulfate-reducing biomass from the 1L enrichment obtained by Meulepas et al. (2009a), to be able to replace the current process at Nyrstar, in which hydrogen is supplied as electron donor for biological sulfate reduction. Once enough CH<sub>4</sub>-oxidizing sulfate-reducing biomass is produced, an operational failure, resulting in biomass wash-out or decay, could set the operation a few years back.

Alternatively, large amounts of AOM biomass could be sampled from the seafloor and used as inoculum for full-scale bioreactors. The highest AOM rate of a natural AOM enrichment is 8–21  $\mu\text{mol g}_{\text{dw}}^{-1} \text{ day}^{-1}$  (Black Sea microbial mats; Treude et al. 2007). At least 4,100 ton dry weight sediment would be needed to replace the current sulfate reduction process; this is from a technological, economical and ecological point of view undesirable. Thus, for biotechnological applications it is essential that CH<sub>4</sub>-oxidizing sulfate-reducing biomass can be grown much faster. Three approaches to obtain faster growth rates are discussed below.

### 5.1 Other inocula

One straight forward approach is to inoculate bioreactors with more promising AOM inocula than the inocula that have been used so far, e.g. Black Sea microbial mats or sediments from thermophilic CH<sub>4</sub> seeps. Black Sea microbial mats form the most active natural AOM inocula, the dissolved methane concentrations below the microbial mats can reach up to 85 mM (Wallmann et al. 2006). Possibly, the relative high conversion rates and dissolved methane concentrations are related to faster maximum growth rates.

Kallmeyer and Boetius (2004) reported that the AOM rate in Hydrothermal Sediment was maximal between 35 and 90°C. Possibly thermophilic anaerobic methanotrophs can also grow faster than ANME from cold-seeps. It would be worth to investigate the growth of the microorganisms, mediating AOM coupled to SR, sampled at a thermophilic “Lost city” site (Boetius 2005).

### 5.2 Other incubation techniques

A second approach is to test novel incubation techniques to enrich the microorganisms responsible

for SR coupled to AOM, e.g. hollow-fiber bioreactors, continuous high-pressure bioreactors or microbial fuel cells. Hollow fibers are semi-permeable tubes, via which for example CH<sub>4</sub> can be supplied to microorganisms growing in a biofilm on the fiber. At the other site of the semi-permeable tube, the sulfate containing liquid phase can be recirculated and refreshed. Diffusion distances in such system are minimal and the shear forces are low compared to gas-lift bioreactors or membrane bioreactors. High shear forces might hamper the formation of CH<sub>4</sub>-oxidizing sulfate-reducing biofilms.

The methane partial pressure positively affected the AOM rate (Nauhaus et al. 2002; Krüger et al. 2005; Kallmeyer and Boetius 2004; Meulepas et al. 2009a) and the Gibbs free energy change of AOM coupled to SR (Valentine 2002). Therefore, the growth of the AOM mediating microorganisms is expected to be faster at elevated CH<sub>4</sub> partial pressures. Although, high pressure bioreactors might not be practical for waste water treatment, they might be ideal to grow sludge as long as a high methane partial pressure can be combined with biomass retention and sulfide removal. Deusner et al. (2009) demonstrated AOM in continuous high pressure bioreactors.

It has been suggested that electrons are transferred from ANME to SRB via extracellular redox shuttles (Widdel and Rabus 2001; Wegener et al. 2008), via membrane bound redox shuttles or so called “nanowires” (Reguera et al. 2005; Stams et al. 2006; Thauer and Shima 2008; Wegener et al. 2008). If this is indeed the case, the methane oxidizers could selectively be grown on a methane-fed anode and the involved sulfate reducers on a sulfate-fed cathode of a microbial fuel cell.

### 5.3 Growth on alternative substrates

A third approach is to grow anaerobic methanotrophs on alternative substrates. A sulfate-reducing CH<sub>4</sub>-oxidizing enrichment was able to utilize thiosulfate and sulfite as alternative electron acceptor for sulfate (Meulepas et al. 2009b), and acetate, formate, carbon monoxide and hydrogen as alternative electron donor for CH<sub>4</sub> (Meulepas 2009c). Given the larger Gibbs free energy change of these conversions, compared to AOM coupled to SR, higher growth rates can be expected on those substrates. If the same microorganisms are involved in both these alternative conversions



and AOM coupled to SR, they could probably be enriched faster on those alternative substrates.

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